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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 3: A61K 39/35, 39/36; C08H 1/00; A23G 1/00; A23F 5/18; A23J 1/14	A1	(11) International Publication Number: WO 82/01132 (43) International Publication Date: 15 April 1982 (15.04.82)

(21) International Application Number: PCT/US81/01353

(22) International Filing Date: 7 October 1981 (07.10.81)

(31) Priority Application Number: 195,271

(32) Priority Date: 8 October 1980 (08.10.80)

(33) Priority Country: US

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(81) Designated States: DE, GB, JP.

Published
With international search report

(54) Title: LOWER ALLERGENICITY AND LOWER THROMBOGENICITY FOODSTUFF AND PROCESS FOR PREPARING THE SAME

(57) Abstract

Foodstuffs of lower allergenicity and lower thrombogenicity are prepared by removing a water-soluble glycoprotein which occurs therein, the glycoprotein being allergenic and thrombogenic. In a preferred processing sequence, the foodstuff is subjected to extraction at a pH above about 4.5, and the resulting supernatent subjected to isoelectric extraction by lowering the pH to between about 4.5 and about 4.0, whereby the glycoprotein precipitates. The glycoprotein precipitate is removed and, if desired, the supernatent, with optional pH adjustment to its starting pH, is returned to the foodstuff residue. The use of glycoprotein in appropriate dosages to desensitize atopic individuals who exhibit hypersensitivity to common allergens is also described.

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LOWER ALLERGENICITY AND LOWER THROMBOGENICITY FOODSTUFF AND PROCESS FOR PREPARING THE SAME

BACKGROUND OF THE INVENTION

It is known that many individuals suffer a severe allergic reaction upon consumption of certain foodstuffs, e.g., coffee and chocolate products. Methods of producing what is typically termed soluble or instant coffee are well known in the art. Such methods generally involve a percolation step, typically a countercurrent percolation step, wherein soluble coffee solids are solubilized into an aqueous extraction liquid, generally water. While many procedural variations have been described in the art, in accordance with the present invention it has been found that such an aqueous extract can be appropriately treated to remove an allergenic, thrombogenic glycoprotein therefrom to provide a coffee of reduced allergenicity and reduced thrombogenicity.

Typical of prior art dealing with the production of soluble coffee are the following discussed patents, all of which are hereby incorporated by reference as teaching methods of producing soluble coffee wherein the present invention finds application, unless otherwise indicated.

U.S. Patent 2,309,884 Bresnick relates to treating products such as chocolate, cocoa, coffee or peanut butter to remove phosphatides therefrom prior to roasting.

U.S. Patent 3,997,685 Strobel deals with obtaining various aroma and flavor products from substrates bearing the same, typically ground, roast coffee. In one step, ground, roast coffee is contacted with cold, wet steam whereby water-soluble constituents are essentially leached from the ground, roast coffee and, following separation and collection of flavor and

aroma concentrates, are eventually condensed to obtain what Strobel et al characterize as a liquid flavor concentrate. Such a liquid flavor concentrate, and later products obtained per Strobel et al which comprise an aqueous system can be subjected to the process of the present invention.

U.S. Patent 4,006,263 Klug et al discloses a method for removing polyhydroxy phenols and polyhydroxy phenol-polysaccharide materials from a coffee extract which react with elemental iron to form an unpleasing precipitate. The coffee extract which is processed per Klug et al to permit precipitation of iron reactive compounds can be subjected to the processing of the present invention to remove glycoprotein therefrom before, during or after the processing of Klug et al, i.e., while present as an aqueous system.

U.S. Patent 4,081,561 Meyer et al relates to a process of producing partially decaffeinated soluble coffee. At numerous stages in the Meyer et al process an aqueous extract, which may be dilute or relatively concentrated, exists; such aqueous extracts can be processed per the present invention to remove glycoprotein therefrom.

U.S. Patent 4,088,794 Katz et al is an improvement upon classical coffee extraction processes which are semi-continuous, counter-current extractions of soluble coffee solids from roasted and ground coffee using an aqueous extraction liquid, Katz et al performing extraction at a specified liquid velocity in a first stage extraction column of at least 0.50 ft/min; extracts as are obtained in Katz et al can be processed per the present invention to remove glycoprotein therefrom.

U.S. Patent 4,092,436 MacDonald et al analyzes in detail various considerations involved in "prewetting" a charge of roast, ground coffee to, inter

alia, eliminate the problem of excessive pressure drops in both steam and aqueous extraction operations. The product of an aqueous extraction as performed in MacDonald et al can be subjected to processing per the present invention to remove glycoprotein thereform.

U.S. Patent 4,100,306 Gregg et al discloses a method of making an improved soluble coffee comprising, inter alia, one or more procedural steps which result in the obtaining of a volatiles-laden extract and an aqueous coffee extract; the volatiles-laden extract is formed by contacting a frost with at least an equal weight of aqueous coffee extract; aqueous coffee extracts or aqueous systems containing the same obtained following the procedure of Gregg et al can be processed per the present invention to remove glycoprotein therefrom.

U.S. Patent 4,129,665 Clark relates to improvements on the classical liquid extraction process used with roast, ground coffee. The process of the present invention can be applied to liquid extracts obtained per the teaching of Clark to remove glycoprotein therefrom.

The present invention, as indicated, also finds application in the formation of chocolate and cocoa of reduced allergenicity and thrombogenicity. While many processes relating to the formation of chocolate and cocoa do not necessarily involve an aqueous extraction, the present invention modifies the same by including an aqueous extraction step which is followed by, or substantially simultaneous with, glycoprotein precipitation.

For example, U.S. Patent 3,997,680 Chalin discloses a method of dutching cocoa; the process of Chalin can be modified so that when the intermediates of Chalin are contacted with an aqueous solution, sufficient aqueous solution is utilized to extract

glycoprotein therefrom, the supernanent is withdrawn and glycoprotein precipitated therefrom, whereafter, if desired, the supernanent is returned to the initial precipitate.

U.S. Patent 4,078,093 Girsh discloses hypoallergenic chocolate; Girsh differs substantially from the present invention in involving the use of a heat treatment which denatures substantially all protein allergens which cause chocolate allergies. The glycoprotein of the present invention is, however, heat-resistant and if processed at the conditions of Girsh, would not be denatured. The present invention is thus based upon a discovery substantially different from that of Girsh.

As earlier indicated, certain individuals are genetically predisposed to allergic reaction upon contact with substances which do not produce any such responses in normal people. These substances comprise a particular class of antigens, or substances capable of provoking an immune response, called "allergens." Thus, allergens elicit the production of immunoglobulin proteins in genetically predisposed or "atopic" individuals, which proteins in turn mediate a number of clinical disorders, including allergic asthma, allergenic rhinitis (hay-fever) and urticaria (hives).

All immunoglobulin proteins are composed of similar basic structural units, each subunit having four polypeptides chains which themselves are distinguishable into two groups, depending upon molecular weight. The polypeptide chains with molecular weight of about 50,000 are denoted "heavy-chains" or H-chains, while chains with molecular weight of approximately 20,000 are "light-chains" or L-chains.

The ability of immunoglobulins to function as antibodies in response to specific antigenic stimuli resides in the H-chain constituent of the immunoglobulin molecule. Five different types of H-chains have

been recognized, and together they define the antigenic classes to which all immunoglobulin molecules belong. Thus, an immunoglobulin protein can be classified as either an IgA, IgD, IgE, IgG or IgM, depending on the type of H-chain it carries. In addition, refined antigenic analysis of human immunoglobulins can distinguish four sub-types of IgG, two sub-types of IgA, and two sub-types of IgM.

When the mucosal and other tissues of an atopic individual come into contact with an allergen, immunoglobulin antibodies of the IgE class and of the IgG_4 class, or "reagins," are produced. Such reaginic antibodies display affinity for the plasma membrane of mast-cells, blood neutrophiles and blood basophiles. Cells which bind the reagins can become sensitized to the allergens which induced the production of the Ig antibodies. Cells that are sensitized in this fashion will rupture and undergo degranulation immediately upon subsequent contact with the allergen, the result being the release from the degranulating cells of one or more vasoactive mediator substances, such as histamine, or precursors, thereof. The release of such a mediator substance by reagin-sensitized cells upon contact with allergens is believed to underlie many atopic responses in hypersensitive individuals.

Since histamine is a common vasoactive mediator substance in atopic individuals, antihistaminic drugs can be effective in the treatment of histamine-mediated allergic states. However, because other mediators may be important to particular atopic conditions, such as asthma and eczema, antihistamines are generally ineffective in combating such conditions.

Alternatively, topical or systemic vasoconstrictors, such as α-adrenergic agents, are employed to reverse the physiological action of histamine and

other vasoactive mediators on the vasculature of mucousal tissue. For example, topical vasoconstrictors taken intranasally as nose drops can be effective in shrinking inflammed nasal mucous, a common symptom of allergic rhinitis.

However, systemic decongestants, which act by non-specific vasoconstriction, may effect an increase in systemic blood pressure which is contraindicated in hypertensive patients. Moreover, topical vasoconstrictors, while more specific in their effects, are often habituating and nearly always capable of producing a "rebound" response, wherein delayed vasodilation follows the initial vasoconstriction.

An alternative to antihistamine therapy and to the use of vasoconstrictors, both topical and systemic, is an immunologic treatment called "desensitization" (This term is used interchangeably with "hyposensitization.") During desensitization, the patient is serially exposed to minute amounts of an allergen or an allergen-containing extract, with the dosage of each exposure gradually increased over a period extending several months. The regimen of allergenic exposures is thought to stimulate the production of IgG (and perhaps IgM and IgA) antibodies reactive with the specific antigen. The antibodies are available thereafter to react with the antigen before it is bound by the reaginic IgE antibodies. Such antibodies are referred to as "blocking antibodies" because they block the action of reagins.

Conventional desensitization treatments have several drawbacks. First, the effectiveness of the desensitization treatment is critically dependent upon using the antigen or antigens responsible for the allergic symptoms. However, selection of the appropriate antigens in this context can be difficult, and it may not be possible to isolate in relatively

purified form the antigen or antigens of interest. Consequently, the only desensitizing material available is often an extract containing the desired antigen(s) in unknown, often highly diluted concentrations in a mixture with other, unspecified allergic factors also of unknown concentration. The effectiveness of such an extract in inducing the synthesis of blocking antibodies that are reactive with the antigen(s) causing the symptoms is therefore reduced.

Moreover, injection of extracts may be dangerous, since deleterious reactions to unidentified constituents may result. Thus, immunologic desensitization must normally proceed under the direction of a highly-trained allergist and therefore cannot be considered as part of routine general practice.

SUMMARY OF THE INVENTION

One object of the present invention is to provide food products of reduced allergenic and thrombogenic characteristics obtained by removing certain allergenic and thrombogenic water-soluble glycoproteins therefrom, and a method for obtaining the same. In one aspect, the present invention finds particular application with coffee and cocoa-type products, most especially in the production of soluble coffee of reduced allergenic and thrombogenic activity.

Another object of this invention is to provide a new group of glycoprotein allergens that may be advantageously employed in desensitizing atopic individuals.

Yet a further object of this invention to provide a desensitization treatment method with improved effectiveness against a broader range of allergenic substances.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

It has been discovered that one or more glycoproteins, later characterized in detail, exists in

tobacco leaf and cigarette smoke condensate to which approximately one-third of human volunteers exhibit immediate cutaneous hypersensitivity. Moreover, glycoproteins which are functionally similar to TGP have been isolated from coffee and chocolate and from ragweed pollen, and have been shown to be immunologically cross-reactive with TGP in experimental animals. It is reasonable to expect that these antigens, like TGP, would also trigger a high incidence of hypersensivity reactions in humans. These one or more glycoproteins (hereafter often merely "glycoprotein" for brevity) further contain polyphenol haptens which activate the factor XII (Hageman factor) dependent pathways of coagulation, fibrinolysis, and kinin generation in normal human plasma. Thus, if such glycoprotein could be removed from food products, substantial benefits would be encountered by sensitive individuals.

The one or more glycoproteins found to exhibit the above potentially harmful effects are water-soluble, have a molecular weight of about 18,000 to about 40,000 daltons, carry one or more haptens which activate factor XII dependent pathways in normal human plasma, have an isoelectric point of about 4 to about 5.8 and precipitate from aqueous solution at a pH of about 4.5 to about 4.0.

It is probable that during removal of said glycoproteins per the present invention fragments thereof having a molecular weight less than about 18,000 are also removed. Since an allergenic response is typically generated by materials having a molecular weight of greater than about 18,000-20,000 daltons, these fragments are probably not allergenic, but rather, in a manner similar to the polyphenol haptens, are only thrombogenic. Polyphenol activators of factor XII are discussed in detail in Ratnoff, O.D., and J.D. Crum,

"Activation of Hageman factor by solutions of ellagic acid," J. Lab. Clin. Med. 63:359 (1964). See also Becker, C.G., and T. Dubin, "Activation of factor XII by tobacco glycoprotein," J. Exp. Med. 146:457 (1977). Those attached to the glycoproteins herein have rutin-like or quercetin-like activity.

Compounds of a molecular weight less than about 18,000-20,000 daltons do not generally have a sufficient size to bridge IgE. Thus, while the glycoprotein can be characterized as an antigen or allergen which is also thrombogenic, the fragments thereof -- and the polyphenol haptens -- are more correctly characterized only as thrombogenic. Hereafter, for brevity, the term "glycoprotein" includes the one or more glycoproteins removed per the present invention, fragments thereof removed per the present invention and attached polyphenol haptens removed per the present invention, albeit results to date indicate the fragments or polyphenol haptens are only thrombogenic.

The glycoprotein is highly allergenic and thrombogenic and can be characterized as an antigen or allergen, and can be positively characterized by a number of test procedures, later described in detail, which can be summarized as follows:

(1) Activation of factor XII dependent pathways in normal human plasma, including coagulation, fibrinolysis and kinin generation as described by Becker, C.G., and T. Dubin, "Cross reactivity and factor XII activation by tobacco, ragweed, and chocolate allergens," Circulation 60(4): II-272 (1979);

(2) Anodic electrophoretic migration in polyacrylamide gel in an alkaline buffer system as described in Becker, C.G., et al., "Hypersensitivity to tobacco antigen," Proc. Natl. Acad. Sci. USA 73(5):1712 (1976);

(3) Reaction with rabbit antibodies directed against the glycoprotein as demonstrated by

hemagglutination inhibition assay (HIA) as described in Becker, et al., Ibid.

(4) By reaction with rabbit antibodies directed against the polyphenol haptens thereof, as demonstrated by HIA (see Becker, C.G. and T. Dubin, "Activation of factor XII by tobacco antigen," J. Exp. Med. 146:457 (1977));

(5) By inducing passive cutaneous anaphylaxis utilizing rabbit, or other animal species, which results in the generation of antibodies directed against the glycoprotein, as described by Becker, C.G., et al., "Induction of IgE antibodies to antigen isolated from tobacco leaves and from cigarette smoke condensate," Am. J. Pathol. 96(1):249 (1979).

This unique set of characteristics characterizes the glycoprotein(s) of the present invention.

The present invention is generally applicable to removing glycoprotein from food products where the food products are amenable to aqueous extraction of the glycoprotein. However, the present invention finds most particular application in removing glycoprotein from soluble coffee, i.e., instant coffee, since glycoprotein occurs therein in substantial amounts and commercial processings used to form soluble coffee are easily modified to include the processing of the present invention without excess capital investment.

In a similar fashion, the present invention can be applied to remove glycoprotein from chocolate and cocoa products, albeit typically with some additional capital investment, since many processes for forming chocolate or cocoa do not generally involve a water extraction step like that employed in many commercial methods for obtaining soluble coffee. However, U.S. Patent 3,778,519 Taralli et al relates to the manufacture of cocoa wherein one step involves treatment

with an aqueous fluid such as water or an alkaline solution; the glycoprotein removal procedure of the present invention could be integrated with the Taralli et al process. Similarly, U.S. Patent 3,754,928 Haney relates to a process for the preparation of dutched cocoa. An alkali treatment is involved and the Haney process could be easily modified to include the glycoprotein removal of the present invention.

As will be apparent to one skilled in the art, the process of the present invention can be applied to food substrates in general which contain glycoprotein; however, since the present invention finds most particular application in removing glycoprotein from coffee and cocoa, hereafter for illustrative purposes the present invention will be discussed in terms of such removal.

In broadest aspect, the present invention can be applied to any aqueous system which results from extraction of the glycoprotein from a food substrate. However, since aqueous mixtures may be difficult to process effectively, it is preferred in accordance with the present invention that the aqueous system which is processed to remove glycoprotein therefrom be a solution or a very dilute mixture, and most typically a solution is involved wherein glycoprotein and other soluble constituents are present. Thus, for simplicity, hereafter an aqueous solution, more particularly an aqueous coffee extract, is generally discussed.

Following extraction of roast, ground coffee by conventional procedures in the art exemplified in, but not limited to, the patents heretofore discussed, glycoprotein is precipitated in accordance with the present invention by isoelectric precipitation, involving reducing the pH of the aqueous coffee extract to a pH in the range of about 4.5 to about 4.0. It

has been found that when the pH is reduced to this range the glycoprotein will precipitate, but precipitation of other desirable factors does not appear to occur, based on results to date.

The resulting glycoprotein precipitate can be removed by conventional means, for example, centrifuging, filtration, and the like, all of which can be practiced in a conventional manner for precipitate removal. The remaining supernatent free of glycoprotein thereafter can be subjected to conventional processing steps, for example, freeze drying, spray drying or the like, whereby the soluble coffee product is obtained. Alternatively, where a first separation is performed from a foodstuff to attain a foodstuff residue and a supernanant, which supernatant is subjected to glycoprotein removal per the present invention; after separation of the glycoprotein precipitate, the supernatant can be recycled to the foodstuff residue in those instances where, for example, the supernatent from which glycoprotein has been removed contains desirable texturing or flavor components.

The present invention thus is predicated upon two points: first, the recognition of the presence of allergenic and thrombogenic glycoprotein in food substrates, as typified by coffee and cocoa beans; second, the discovery that the glycoprotein can be removed therefrom to provide a product of reduced allergenicity and reduced thrombogenicity.

For example, cocoa can be produced by a number of processes, with one typical process being the well-known dutching process. In this process, after roasting the beans and removing the husk or shell, the remaining pieces of cocoa beans, commonly referred to as nibs, are crushed or ground to break the cells and form a smooth, creamy paste known as a chocolate liquor. The liquor is typically fed to a hydraulic

press where a press cake is formed by removing a part of the fat or cocoa butter. The press cake then can be broken and mixed with an alkalizing solution in a pressure cooker and, after vacuum drying, the resulting cooled product is ground to a fine powder which can be used in various chocolate products.

While the alkalizing step in such a conventional process involves relatively low moisture contents, e.g., on the order of 20-35%, such a procedural step can be converted into an extraction step by utilizing greater amounts of water. Thus, glycoprotein can be extracted at an alkaline pH from the supernanant which results, the supernatant being removed, the pH being reduced to a pH on the order of about 4.5 to 4.0 using any common acid, for example, hydrochloric acid, the glycoprotein precipitated and removed by means as earlier described, and then, if desired, the supernanant returned to the original cocoa precipitate. Thereafter, processing is in a conventional fashion except, of course, the cocoa is free of the glycoprotein described herein.

As earlier indicated, the extraction conditions used to obtain an aqueous system containing the glycoprotein which is subjected to the glycoprotein removal of the present invention are generally in accordance with prior art procedures as earlier exemplified for forming, e.g., coffee extracts, cocoa extracts and the like, since the glycoprotein removal of the present invention is, for economic reasons, merely introduced as a unique processing step in conventional foodstuff preparation procedures. Foodstuff extraction should not in most instances occur at a pH below about 4.5 since, in this instance, glycoprotein precipitation would be initiated and possibly glycoprotein would undesirably be retained in solid constituents which might be desirable for inclusion in the end product

foodstuff. In those instances, of course, where only water soluble constituents of the foodstuff are to be retained, theoretically extraction can be effected simultaneously with the glycoprotein removal. However, where solid constituents of the foodstuff are to be present in the end product foodstuff, extraction should be at a neutral, or more preferably, alkaline pH so that glycoprotein is not retained in any solid constituent, but rather is retained in the liquid extract phase for subsequent removal and, if desired, recycling of the supernanant free of glycoprotein.

It is to be noted that, as heretofore indicated, theoretically extraction can occur at any pH above about 4.5. However, results to date indicate that most preferred extraction rates and removal of glycoprotein occur at a neutral to alkaline pH, e.g., 8.5. Accordingly, the glycoprotein removal step of the present invention finds particular application in the processing of foodstuffs which are extracted to yield a liquid extract of a neutral or alkaline pH, most preferably a pH on the order of 8.5 or above.

The processing conditions of the present invention are not overly critical and can be freely varied by one skilled in the art. For example, processing pressure seems to be relatively unimportant, with processing typically being at normal atmospheric pressure. There is no reason in theory, however, why processing cannot be at sub- or super-atmospheric pressures, though results to date indicate that no benefits accompany processing at other than atmospheric pressure.

The temperature of processing is also relatively unimportant, and processing is typically at ambient temperature.

Although prior art extraction conditions can in general be followed, results to date indicate that

extraction of glycoprotein is also effective at higher temperatures, for example, temperatures up to the boiling point of the aqueous system being used. Again, however, there is no prohibition against processing at lower temperatures or processing at temperatures up to and including the boiling point of the system, although no substantial benefits apparently are obtained in improving glycoprotein removal by processing at sub- or super-ambient temperatures.

The time of processing is also not overly important, since results to date indicate that glycoprotein precipitates substantially instantaneously once the requisite pH is reached, i.e., at a pH on the order of about 4.5 to about 4.0. In this regard, as the acid is added glycoprotein immediately becomes visible as a cloudy precipitate in the system.

The amount of water used to form an aqueous system is, in a similar fashion, not overly important, albeit sufficient water should be used to ensure adequate removal of glycoprotein from the food substrate being processed; such amounts can easily be selected by one skilled in the extraction art.

While this is currently the most preferred way of removing the glycoproteins, applicable alternative procedures exist for removing the glycoprotein, for example, precipitation with ammonium sulfate, and electrophoretic separation. Ammonium sulfate is, however, often difficult to remove from foodstuffs, and electrophoretic separation involves costly equipment.

A glycoprotein has also been extracted from tobacco leaves which has been found to elicit immediate cutaneous hypersensitivity in certain human subjects. This "tobacco glycoprotein" (TGP), when injected intracutaneously, resulted in the immediate development of wheal and flare reactions, indicative

of cutaneous hypersensitivity, in a substantial number of human subjects. See Becker, C.G., et al., Proc. Natl. Acad. Sci. USA, loc. cit. (1976).

TGP apparently activates the humoral immunological system by triggering a specific, immunoglobulin-mediated release of histamine or other inflammatory mediators which induce myocardial arrythmias, as described by Levi, R, et al., "Cardiac and pulmonary anaphylaxis induced by tobacco glycoprotein (TGP)," Fed. Proc. 37(3): 590 (1978), and which may induce arteriosclerosis in hypersensitive individuals. See Becker, C.G. and T. Dubin, "Tobacco allergy and cardiovascular disease," Cardiovascular Medicine 3(8): 851 (1978). In addition, TGP has been shown to activate clotting factor XII in human plasma, resulting in the generation of clotting, fibrinolysis and kinin activity. See Becker, C.G. and T. Dubin, J. Exp. Med., loc. cit. (1977).

It now has been discovered that coffee and cocoa, as well as ragweed pollen contain allergens with chemical, immunologic and functional characteristics that are similar to TGP. Thus, antigens from cocoa (GP-Coc) coffee (GP-Cof) and ragweed pollen (GP-RW) can be isolated which are immunologically cross-reactive with TGP, and like TGP are capable of activating factor XII-dependent pathways in human plasma.

Further, individuals hypersensitive to cocoa- or coffee-derived foodstuffs, to ragweed and other plant pollens, and/or to a number of other common allergenic substances may be able to be routinely and effectively desensitized by successive exposures to TGP or to the other antigens based on their structural similarity. Surprisingly, TGP thus can be employed to counter atopic symptoms elicited by allergenic substances wholly unrelated to tobacco without the problems

related to antigen characterization and isolation described above.

In another aspect, the present invention therefore provides a method of desensitizing individuals to a wide class of allergens, which is predicated broadly upon the discovery that individuals sensitive to, for example, TGP, ragweed, GP-Cof, GP-Coc, etc., can be densensitized to this entire class of allergens by appropriate treatement with merely only one member of the class. In a preferred embodiment, a sensitive individual is desensitized to this class of allergens by treatment with GP-Cof and/or GP-Coc, and in another preferred embodiment individuals sensitive to GP-Cof and/or GP-Coc and/or ragweed pollen are desensitized thereto by treatment with TGP.

Example 1

This example illustrates not only the extraction of the glycoprotein from coffee but the further purification and testing of the glycoprotein.

Initial extraction of coffee antigen from Chock-Full O'Nuts Coffee (Candler Coffee Corp., N.Y.C.) was performed with a West Bend coffee machine (West Bend Co., West Bend, Wis.) according to the manufacturer's instruction for preparing coffee.

For extraction purposes, the above procedure is equivalent to a typical extraction as is used in the commercial production of soluble (instant) coffee. The coffee was clarified by centrifugation at 20,000 × g and extracted twice with equal volumes of petroleum ether. The infranate was then extracted twice with equal volumes of ethyl ether, whereby hydrocarbon-soluble components in the coffee were removed. This step is only necessary where one desires to purify the glycoprotein itself, and initial clarification and hydrocarbon extraction are not necessary where the objective is merely to remove the glycoprotein.

The infranate was retained and granular $(NH_4)_2SO_4$ was added to 50% saturation. This result is an initial precipitation of glycoprotein, and is typically practiced only where the glycoprotein itself is to be purified. The precipitate was collected by centrifugation and redissolved in phosphate buffered physiologic saline, pH 7.4 (PBS). This solution was again clarified by centrifugation and reprecipitated with $(NH_4)_2SO_4$ at 50% saturation; this precipitation is typically only practiced where it is desired to purify the glycoprotein itself. The precipitate was then redissolved in PBS to a concentration of approximately 4 mg/ml of protein. The pH was lowered to 4.0 by dropwise addition, with stirring, of 0.1N HCl; a large quantity of precipitate formed which was collected by centrifugation. On a commercial scale, it is contemplated that isoelectric precipitation using an acid such as HCl would be the only precipitation performed, since this appears to be the most efficient precipitation technique. If it is desired to later return the pH of the supernanent after glycoprotein removal to an alkaline pH, such can be accomplished using low cost base materials such as, e.g., NaOH. Of course, acids other than HCl and bases other than NaOH can be used with success so long as the constituents of the foodstuff being processed are not adversely affected. The precipitate was found to contain the glycoprotein. For foodstuff processing, the supernate could be recycled to the starting foodstuff at this stage, with or without pH adjustment as desired, or the same can be discarded.

The glycoprotein was then further purified as follows. Isoelectric precipitation of the glycoprotein from PBS using HCl as above described (pH 4.0-4.5) was repeated three to four times until the supernate was essentially colorless. The final

precipitate was dissolved in 3 ml of PBS and applied to a 2.5 × 100 cm column of Biogel P-10 equilibrated with PBS and then washed with PBS. The brown, void volume peak was collected. The glycoprotein was then concentrated by isoelectric precipitation at pH 4.0 with HCl as above described. The precipitate was collected by centrifugation and redissolved in Tris-barbital buffer at pH 8.9 and applied to an alkaline polyacrylamide gel column, and preparative continuous flow electrophoresis was performed as described by Becker, et al., Proc. Natl. Acad. Sci. USA, loc. cit. (1976). The glycoprotein emerged as a sharp, highly anodic peak. This was again concentrated by isoelectric precipitation at pH 4.0 with HCl as above described or by pressure dialysis using an Amicon PM-10 membrane. The precipitate was then redissolved in PBS and applied to a 2.5 × 40 cm column of Biogel P-150 equilibrated with PBS. The column had previously been calibrated with proteins of molecular weight 1×10^6, 165,000, 67,000, 45,000, 25,000, 17,800 and 12,400 daltons. The column was washed with PBS and a broad brown peak emerged in fractions corresponding to an apparent molecular weight on the order of 20,000-40,000 daltons. The midpoint of this peak corresponded to a molecular weight of 26,000 daltons.

Tubes containing the 20,000-40,000 molecular weight material were pooled, dialyzed exhaustively against distilled water and lyophylized in a conventional manner.

Example 2

Initial extraction of glycoprotein from cocoa powder was performed by dissolving Hershey's Cocoa Powder (Hershey Food Corp., Hershey, Pa.) in boiling water according to the manufacturer's instructions for preparing "hot chocolate".

Thereafter, the glycoprotein was purified as described in Example 1.

Example 3

Ragweed pollen was obtained from Greer Laboratories, Lenoir, N.C. The pollen was pulverized and the glycoprotein extracted therefrom as described in Example 1.

The above glycoproteins, hereinafter referred to respectively as GP-Cof (Example 1), GP-Coc (Example 2), and GP-RW (Example 3), were then tested per the following procedure, along with TGP isolated and purified from tobacco leaf according to the protocol of Becker, et al., Proc. Natl. Acad. Sci. USA, loc. cit. (1976).

Glycoprotein Quantification

Because of the large number of precipitation and chromotographic procedures used in purification of the glycoprotein from the above sources, final yield did not accurately reflect content. Content was estimated by measuring the quantity of glycoprotein present after lipid extraction, precipitation with $(NH_4)_2SO_4$ and isoelectric precipitation (as described above), by hemagglutination inhibition assay employing tanned human erythrocytes coated with TGP and capable of being agglutinated by rabbit antibodies to TGP or to rutin BSA conjugates, as described above.

Expressed as µg or mg per gm of starting material, the following results were obtained:

Starting Material	Glycoprotein Content
TGP	5 mg/gm
Cocoa	685 µg/gm
Coffee	12.2 mg/gm
GP-RW	425 µg/gm

Electrophoretic/Isoelectric Focusing Analysis

TGP, GP-Cof, GP-Coc and antigens from ragweed pollen (GP-RW) were then compared by electrophoresis and isoelectric focusing.

TGP, GP-Cof, GP-Coc and RW were compared by electrophoresis on alkaline 7.5% polyacrylamide gels according to the method of Davis and Ornstein, described in Methods in Immunology and Immunochemistry, Vol. II, C.A. Williams and M.W. Chase (eds.), Academic Press (N.Y.), 1968, pages 38-47. Following electrophoresis the gels were fixed in methanol: acetic acid: water (40:10:50:) and stained with either Coomassie Brilliant Blue or the PAS reaction. These antigens were also compared by isoelectric focusing in 7.5% polyacrylamide gels which were made 1.5% in pH 3-10 ampholine and 0.5% in pH 4-6 ampholine (LKB, Plainview, N.Y.), as described by Williamson in Chapter 8 of Handbook of Experimental Immunochemistry, D.M. Weir (ed.), Blackwell Scientific Publications (Oxford), 1973. These gels were fixed and washed to remove ampholines, and stained.

TGP, GP-Cof, GP-Coc and RW were found to migrate identically in alkaline 7.5% polyacrylamide gels resulting in bands which were PAS positive and (unstained) providing gels which were brown - GP-Cof, red-brown - GP-Coc or yellow-brown - RW.

When TGP, GP-Cof, GP-Coc and GP-RW were subjected to isoelectric focusing in 7.5% acrylamide gels containing a mixture of pH 3-10 and pH 4-6 ampholytes, an array of similar closely spaced bands were formed with isoelectric points between pH 4.0-5.9. These bands are also PAS positive and in unstained preparations were TGP-brown - GP-Cof; red-brown - GP-Coc; or yellow-brown - RW, as described above.

Effect of Glycoproteins on Activation of Factor XII Dependent Pathways:

Partial Thromboplastin Time - The effect of TGP, GP-Coc, GP-Cof and GP-RW on the partial thromboplastin (PTT) of normal human plasma was measured using a fibrometer available from Baltimore Biological Laboratories (Baltimore, Md.), following the procedure of

Becker, C.G. and T. Dubin J. Exp. Med. loc. cit. (1977).

Euglobulin Clot Lysis Time - The effect of these antigens on euglobulin clot lysis time (ECLT) was measured according to the technique of Ogston D., et al., "The assay of a plasma component necessary for the generation of a plasminogen activator in the presence of Hageman factor (Hageman co-factor)," Brit. J. Haematol. 20:209 (1971).

Kallikrein Activity - Normal citrated plasma which had been stored at -70°C was activated at 37°C for 15 minutes with the glycoproteins in concentrations of approximately 200 µg/ml plasma. Twenty volumes of 0.01 M Na acetate buffer, pH 4.8 was added, mixed, allowed to stand for 20 minutes at 37°C and centrifuged for 10 minutes at 10,000 rpm at room temperature. The resulting euglobulin precipitate was dissolved in a volume of PBS equal to that of the original plasma and maintained at 37°C. One hundred microliters of the euglobulin were added to a cuvette (at 37°C) containing 2.3 ml 0.05 M Tris/HCl, pH 7.8, I 0.05 with NaCl and 100 µl α-benzoyl-Pro-Phe-Arg-p-nitroanilide (Vega Biochemicals, Tucson, Ariz.). The absorbance at 405 nm was recorded every minute for 10 minutes in a Zeiss spectrophotometer maintaining the temperature of the cuvette at 37°C. A standard curve of p-nitroaniline dissolved in Tris buffer, linear from 0.5 to 50 n moles, was used as reference. To demonstrate the specificity of this substrate for kallikrein, duplicate assays were set up using lima bean trypsin inhibitor (100 µg) and soy bean trypsin inhibitor (20 µg) incubated for one minute at 37°C prior to addition to the substrate. Plasma from patients genetically deficient in factor XII or in pre-kallikrein were also challenged with these glycoproteins and amidolytic activity measured with this substrate.

TGP, GP-Cof, GP-Coc and GP-RW were all capable of shortening the partial thromboplastin (PTT) and euglobulin clot lysis time (ECLT) of normal human plasma. Addition of the glycoproteins to normal plasma resulted in generation of amidolytic activity as measured by the rate of hydrolysis of the chromogenic substrate Benzyl-Pro-Phen-Arg-nitroanilide. This activity was inhibited or greatly reduced by addition to the plasma of soy bean trypsin inhibitor, but not by addition of lima bean inhibitor, indicating that the amidolytic activity was due to plasma kallikrein. These observations, taken together, indicate that GP-Cof, GP-Coc and GP-RW can activate the factor XII dependent pathways of coagulation, fibrinolysis and kinin generation. These effects could not be demonstrated in plasma from humans genetically deficient in factor XII or in pre-kallikrein. Heating the glycoproteins to 180°C for four hours did not change its capacity to shorten partial thromboplastin time and generate kallikrein activity. Heating E. coli endotoxin completely inhibited its capacity to activate factor XII dependent pathways.

Example 4

To illustrate the cross-reactivity of anti-TGP antibodies with a range of different antigens, TGP was isolated and purified according to the protocol of Becker, et al., Proc. Natl. Acad. Sci. USA, loc cit. (1976).

Adult Hartley strain guinea pigs were then injected with 100 µg of the TGP in alum and similarly boosted after one month. Ten days later they were anesthetized and bled from the retro-orbital sinus. The serum was stored at -70°C. California White rabbits were immunized neonatally with the TGP and boosted subsequently at monthly intervals as described by Becker and T. Dubin, J. Exp. Med., loc. cit. (1976).

Skin sites in adult guinea pigs were sensitized by intradermal injection of 0.2 ml of a 1:00 dilution of guinea pig anti-TGP antibodies. Control sites were injected wth normal guinea pig serum siluted similarly. Eighteen hours later the sensitized animals were injected intravenously with 1 ml of a 1.5% solution of Evans Blue dye. The skin sites were challenged by intradermal injection of 1, 5, or 25 µg of TGP, GP-Coc, GP-Cof, or GP-RW. Thirty minutes later the animals were sacrificed, skinned, and the zones of leakage of Evans Blue measured and photographed.

As shown in Table I, infra, TGP, GP-Coc, GP-Cof, and GP-RW all elicited cutaneous anaphylaxis reactions at skin sites sensitized 24 hours previously with serum from a guinea pig immunized with TGP. These antigens did not trigger anaphylactic responses at sites of injection of normal guinea pig serum, or at unsensitized sites. TGP triggered larger PCA responses than the other antigens tested.

TABLE I

COMPARISON OF ANTIGENS BY PCA IN THE GUINEA PIG

	Antigen Dose (lesion diameter in mm.)		
Antigen Tested	25 µg.	5 µg.	1 µg.
TGP	15	12	9
GP-Coc	11	8	6
GP-Cof	6	3.5	2
GP-RW	5	2	1
PBS	0	0	0

Antigenic challenge of sites treated with normal guinea pig serum produced no response.

Similar observations were made when passive cutaneous anaphylaxis (PCA) experiments were carried out in rabbits.

From the test data set forth in Table I, it can be seen that anti-TGP antibodies contained in the guinea pig serum reacted not only with TGP, but also with the glyprotein extracted from coffee, cocoa and ragweed pollen. This unexpected result indicates that either TGP, GP-Coc, GP-Cof or GP-RW can be employed as the hyposensitizing material to hyposensitize an atopic human patient who is hypersensitive to any or all of the polyphenol hapten-carrying allergens.

The hyposensitizing material could be administered by any of the conventional techniques used by trained allergists, e.g., subcutaneous injection. The dosage levels for the hyposensitizing material are ascertained on a case-by-case basis since the clinical responses to exposure, as is well-known in the field of allergy, differs between subjects.

The desensitizing process may be followed during treatment by monitoring the subject for decreasing allergic sensitivity. Thus, one skilled in the art would appreciate that the progression of treatment could be followed utilizing a standard bioassay technique, such as the wheal-and-flare method, or by quantifying the production of antibodies of different classes using, e.g., a radioimmunoassay technique.

While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof.

CLAIMS

1. A coffee- or cocoa-derived beverage or extract having reduced allergenic and thrombogenic activity from which has been removed one or more glycoproteins having a molecular weight within the range of about 18,000 to about 40,000 and carrying one or more polyphenol haptens which activate factor XII-dependent pathways in normal human plasma, which glycoproteins or fragments thereof percipitate from aqueous solution at between pH 4.5 and pH 4.0.

2. A process for producing coffee and cocoa beverages and extracts having reduced allergenic and thrombogenic activity comprising the removal therefrom of one or more glycoproteins which have a molecular weight within the range of about 18,000 to about 40,000, which precipitate from aqueous solution at between pH 4.5 and pH 4.0, and which carry one or more polyphenyl haptens which activate factor XII-dependent pathways in normal human plasma.

3. A desensitization method comprising the administration of one or more glycoproteins or fragments thereof in a physiologically acceptable carrier medium, the glycoproteins having molecular weights in the range of about 18,000 to about 40,000 daltons, precipitating from aqueous solution at between pH 4.5 and 4.0, and carrying one or more polyphenol haptens which activate factor XII-dependent pathways in normal human plasma, in a medical regimen of successively increasing dosages of said glycoproteins or fragments thereof which is effective in blocking allergenic response in atopic individuals to non-tobacco allergens.

4. A method in accordance with claim 1, wherein said medical regimen is effective in blocking allergenic responses to ragweed pollen and to products derived from coffee or cocoa.

5. A glycoprotein or glycoprotein fragment in substantially purifed form isolated from coffee, cocoa or ragweed pollen having a molecular weight within the range of about 18,000 to about 40,000 daltons, which glycoprotein or fragment undergoes precipitation from an aqueous solution at between pH 4.5 and 4.0 and carries one or more polyphenol haptens that activate factor XII-dependent pathways in normal human plasma.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US81/01353

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [2]

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.[3] A61K 39/35; A61K 39/36; C08H 1/00; A23G 1/00; A23F 5/18; A23J 1/14

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	426/593,594,431,432,433,495; 260/112R,123.5; 424/9,91

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	US,A, 2,309,884, PUBLISHED 02 FEBRUARY 1943,BRESNICK.	1,2
X	US,A, 3,845,220, PUBLISHED 29 OCTOBER 1974,SUZUKI.	1,2
X	US,A, 4,078,093, PUBLISHED 07 MARCH 1987,GIRSH.	1,2
X	US,A, 4,208,440, PUBLISHED 17 JUNE 1980, SCHMIDT.	1,2
X	N, INTRODUCTION TO CLINICAL ALLERGY, PUBLISHED 1973, PUBLISHED BY CHARLES C. THOMAS: SPRINGFIELD, OHIO, SEE PAGES 290,297.	1,2
X	N, COFFEE PROCESSING TECHNOLOGY, VOLUME 2, PUBLISHED 1963, PUBLISHED BY AVI PUBLISHING COMPANY, WESTPORT, CONNECTICUT, SEE PAGES 120,121,163,164,165.	1,2
X	US,A, 1,613,313, PUBLISHED 04 JANUARY 1927, CSONKA.	3-4
X	US,A, 2,500,145, PUBLISHED 14 MARCH 1950,FERGUSON.	3-5
X	US,A, 3,846,937, PUBLISHED 12 NOVEMBER 1974,STABA.	3-5
X	US,A, 3,995,023, PUBLISHED 30 NOVEMBER 1976,NIESCHULZ.	3-5
X	US,A, 3,893,993, PUBLISHED 08 JULY 1975,MULLAN.	3-5
X	US,A, 4,140,679, PUBLISHED 20 FEBRUARY 1979,MALLEY.	3-4
X,P	US,A, 4,269,764, PUBLISHED 26 MAY 1981, PATTERSON.	3-5

* Special categories of cited documents: [15]

"A" document defining the general state of the art

"E" earlier document but published on or after the international filing date

"L" document cited for special reason other than those referred to in the other categories

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but on or after the priority date claimed

"T" later document published on or after the international filing date or priority date and not in conflict with the application, but cited to understand the principle or theory underlying the invention

"X" document of particular relevance

IV. CERTIFICATION

Date of the Actual Completion of the International Search [1]	Date of Mailing of this International Search Report [2]
17 DECEMBER 1981	06 JAN 1982
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	JOSEPH K. GOLIAN

Form PCT/ISA/210 (second sheet) (October 1977)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A,X	N, NATIONAL ACADEMY OF SCIENCE PROCEEDINGS, VOLUME 73, ISSUED MAY 1976, WASHINGTON, D.C., C.G. BECKER, HYPERSENSITIVITY TO TOBACCO ANTIGEN, PP. 1712-1716.	3-5
A,X	N, THE JOURNAL OF EXPERIMENTAL MEDICINE, VOLUME 146, ISSUED 1977, NEW YORK, NEW YORK, C.G. BECKER, ACTIVATION OF FACTOR XII BY TOBACCO GLYCOPROTEIN, PP. 457-467.	3-5

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

I. Coffee or Coffee product or process claims 1-2
II. Desensitization method, claims 3-4
III. Glycoprotein or fragment, claim 5

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.